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Short Communication

Determination of 3′-deamino-3′-[2(*S*)-methoxy-4-morpholinyl]doxorubicin, a new morpholinyl anthracycline, in plasma by high-performance liquid chromatography with fluorescence detection

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ABSTRACT

A sensitive and selective high-performance liquid chromatographic method for the determination of 3′-deamino-3′-[2(*S*)-methoxy-4-morpholinyl]doxorubicin and its possible 13-dihydro metabolite in human plasma has been developed. The plasma samples were buffered and the drugs and internal standard (doxorubicin) were extracted with diethyl ether–*n*-butanol, back-extracted into 0.3 *M* phosphoric acid, then analysed by reversed-phase liquid chromatography. Quantitation was achieved by fluorescence detection of the eluate. The linearity, precision and accuracy of the method were evaluated. No interference from blank plasma sample was observed. The suitability of the method for *in vivo* samples was checked by analysis of plasma samples drawn from female rats that had received repeated intravenous doses of the test compound.

INTRODUCTION

Anthracycline antibiotics are chemotherapeutic agents with significant antitumour activity. Since the discovery of doxorubicin (DXR) and daunorubicin (DNR) (Fig. 1), a large number of analogues of this family have been studied in an attempt to obtain new anticancer agents with improved efficacy and therapeutic index.

Morpholinyl anthracyclines are a special subclass of N-alkyl derivatives prepared from DXR and DNR [1]. In the initial screening, the notable property of these compounds was the considerably lower dose requirement (*i.e.* increase in antitumour potency) encountered both *in vitro* and *in vivo* [1]. 3′-Deamino-3′-[2(*S*)-methoxy-4-morpholinyl]doxorubicin (FCE 23762, I, Fig. 1) is a new derivative of DXR with a modified daunosamine moiety in position 3′, synthesized and studied in the Research and Development Laboratories of Farmitalia Carlo Erba.

This compound is between 3- and 15-fold more potent than DXR *in vitro* and 80- and 120-fold more potent *in vivo* [2]. In addition to its clear activity on multidrug resistant cells and tumours, it was also found to be equally or more effective

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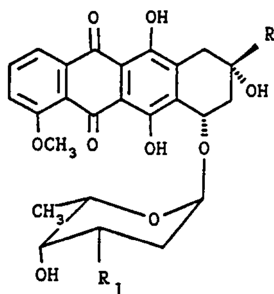

R	R_1	
$-COCH_2OH$	$-NH_2$	DOXORUBICIN
$-CH(OH)CH_2OH$	$-NH_2$	DOXORUBICINOL
$-COCH_3$	$-NH_2$	DAUNORUBICIN
$-CH(OH)CH_3$	$-NH_2$	DAUNORUBICINOL
$-COCH_2OH$	N O OCH_3	FCE 23762
$-CH(OH)CH_2OH$	N O OCH_3	FCE 26176

Fig. 1. Structures of doxorubicin, daunorubicin, doxorubicinol, daunorubicinol, FCE 23762 (I) and FCE 26176 (II).

than DXR in experimental solid neoplasms, whereas it was less active than DXR in other models, such as Lewis lung carcinoma [2].

As reported in a previous study [3], one of the two major mammalian enzyme systems involved in the metabolism of DXR and DNR is the cytoplasmic aldo–keto reductase. The enzymic products of aldo–keto reductase, doxorubicinol and daunorubicinol (Fig. 1), retain some inhibitory activity both *in vitro* and *in vivo* [4,5]. However, anthracyclines with R like doxorubicin (Fig. 1) appear to be reduced to a lesser extent than anthracyclines with R like daunorubicin [6].

Several investigators have successfully employed high-performance liquid chromatography (HPLC) with fluorescence detection for specific and sensitive determination of anthracyclines in biological fluids [7–11]. Therefore our attempts to set up an analytical method for this new compound were directed toward the use of this analytical technique.

In the present study, optimal conditions were developed for the determination of the parent compound (I) as well as its possible metabolite 13-dihydro-3′-deamino-3′-[2(*S*)-methoxy-4-morpholinyl]doxorubicin (FCE 26176, II, Fig. 1) in plasma. The HPLC method developed was fully validated and used in a preliminary determination of the two compounds in plasma samples obtained from rats that had received the drug during a toxicological study.

EXPERIMENTAL

Chemicals and solutions

Compounds I (as HCl salt) and II (as HCl salt) and DXR · HCl (internal standard, I.S.) were supplied by the Chemical Development Department of Farmitalia Carlo Erba. All other chemicals and solvents were analytical grade from Farmitalia Carlo Erba (Milan, Italy).

Stock solutions were prepared by dissolving a weighed amount of each compound in distilled water. From this solution (stable for at least one month if stored at 4°C in the dark) working solutions were prepared daily by dilution with bidistilled water.

All glassware was silanized before use by treatment with a dimethyldichlorosilane–toluene solution (7:93, v/v), followed by double rinsing with absolute ethanol and chloroform, to prevent formation of drug complexes and degradation on the active free silanol groups of the glassware.

Equipment

The HPLC system consisted of a Spectra Physics Model SP 8700 pump (Santa Clara, CA, USA) equipped with a Rheodyne Model 7125

sampling valve with 200-μl loop, a Jasco Model 821-FP fluorimetric detector (Hachioji, Japan) and a Spectra Physics Model SP 4270 integrator. A 1-V signal was sent from the detector to the integrator.

Chromatographic conditions

The chromatographic separation was performed on a 250 mm × 4.6 mm I.D. Hypersil ODS column, particle size 5 μm (Shandon Scientific, Runcorn, UK) with a Survival precolumn packed with Pellicular ODS (particle size 37–53 μm; Whatman, Clifton, NY, USA). The mobile phase was acetonitrile–tetrahydrofuran–0.05 *M* KH_2PO_4 brought to pH 2.7 with 1 *M* H_3PO_4 (18:10:72, v/v). The flow-rate was 1.0 ml/min.

Sample extraction

To 1.0 ml of human plasma, 0.1 ml (10.0 ng) of working I.S. solution was added. The solution was placed in a 10-ml conical glass centrifuge tube and mixed with 0.5 ml of 0.1 *M* borate buffer (pH 8.4). After the addition of 4 ml of diethyl ether–*n*-butanol (9:1, v/v) the tubes were capped and immediately shaken on a rotary mixer for 1 min, then centrigufed at 1200 *g* for 3 min in order to clearly separate the two phases. The upper organic phase was transferred to another tube, and the extraction step was repeated. The collected organic phase was extracted with 0.25 ml of 0.3 *M* phosphoric acid by vortex-mixing for 1 min. After centrifugation as above, the organic phase was discarded and the aqueous phase was washed with 0.5 ml of *n*-hexane by vortexing. Finally, the tube was centrifuged and the *n*-hexane was removed. An aliquot (200 μl) of the aqueous solution was injected onto the column.

Determination of quality control and calibration samples

Analyses of blank human plasma spiked with known amounts of I, II and the I.S. were carried out by applying the above procedure. The linearity was evaluated from six calibration curves prepared and run on six different days in the concentration range 0.5–100 ng/ml for both compounds. The precision and accuracy were evaluated by repeated analyses of the two compounds at three concentrations (0.7, 7 and 70 ng/ml) in five replicate samples analysed on five different days. All chromatograms obtained were evaluated by peak-height measurement.

To evaluate the absolute extraction recovery, the peak height of extracted plasma samples was compared with the peak height obtained with the unextracted standard solution injected directly onto the chromatograph.

Chromatographic system suitability test

On each day, before the analysis of unknown and/or calibration samples, the performance of the chromatographic system was checked in order to ensure that controlled conditions were used in the assay. Three parameters were used to define the suitability of the chromatographic system [12]. The analysis of extracted samples was carried out only if the values of the three parameters checked were within the range of values described below.

Column efficiency. This was evaluated as the number of theoretical plates of the column calculated from the equation $N = 5.54\,(t_R/W)^2$, where t_R is the retention time (mm) of the compound tested and W is the peak width (mm) at half height. The value of N must be at least 3500 for each compound.

Peak symmetry. This was evaluated as symmetry factor S_F, calculated from the equation $S_F = W_{0.05}/2A$, where $W_{0.05}$ is the peak width (mm) measured at 1/20 of the peak height and A is the distance (mm) between the perpendicular dropped from the peak maximum and the leading edge of the peak at 1/20 of peak height. S_F must be 1.1 or less.

Resolution factor. The resolution factors between the peaks of I and II *versus* I.S. were calculated from the equation $R = 2\,(t_2 - t_1)/(w_1 + w_2)$, where t_1 and t_2 refer to the respective retention times, and w_1 and w_2 are their baseline bandwidths (t and w must be measured in the same units, *i.e.* mm or s). R must be greater than 2.0.

TABLE I

EXTRACTION RECOVERY OF THE THREE ANALYTES FROM PLASMA ($n=5$)

Analyte	Concentration added (ng/ml)	Absolute recovery (%)	R.S.D. (%)
I	0.5	93.2	10.9
	10.0	95.1	6.0
	100.0	88.5	9.1
II	0.5	61.5	8.3
	10.0	65.6	3.3
	100.0	69.4	8.0
Doxorubicin	10.0	49.7	6.3

RESULTS AND DISCUSSION

High selectivity and sensitivity were obtained by fluorimetric detection. The UV absorption spectrum of I recorded in the mobile phase in the range 220–600 nm shows an absorption maximum at 506 nm. The emission maximum recorded with an excitation wavelength of 506 nm was at 560 nm. Since DXR and II both gave a very good fluorescent response under these conditions, these wavelength values were used for the detection of all three compounds.

The highest chromatographic selectivity was obtained by using a low concentration of acetonitrile in the mobile phase, and sharp well separated peaks were obtained by adding tetrahydrofuran to the mobile phase as a third component, as was found with other anthracyclines [7,8]. Under the chromatographic conditions chosen, the more lipophilic I was eluted with a t_R of 10 min, close to the possible metabolite and I.S. peaks.

Blank plasma samples from different animal species assayed as described showed no significant peak at the t_R of the compounds of interest.

Previous studies of the determination of other anthracyclines in biological fluids [13,14] showed that the extraction yield was strictly related to the pH of the aqueous phase. We found that the most favourable extraction pH for I and II was

TABLE II

ACCURACY AND PRECISION OF THE METHOD FOR THE DETERMINATION OF I IN PLASMA

Control sample (ng/ml)	Day	n	Accuracy			Precision		
			Mean found (ng/ml)	Found/added (%)	S.D. (%)	R.S.D. (%)		Found/added ($n=25$) (%)
						Intra-day	Inter-day ($n=25$)	
0.7	1	5	0.74	105.4	3.1	2.9		
	2	5	0.70	99.4	11.9	11.9		
	3	5	0.67	96.3	8.1	8.4		
	4	5	0.73	103.7	6.7	6.9		
	5	5	0.72	102.3	4.4	4.2	7.5	101.4
7.0	1	5	7.35	105.0	9.4	9.0		
	2	5	6.80	97.2	9.7	10.0		
	3	5	6.62	94.5	10.5	11.1		
	4	5	7.15	102.1	7.4	7.3		
	5	5	7.09	101.3	4.1	4.1	8.4	100.0
70.0	1	5	67.57	96.5	2.1	2.2		
	2	5	67.69	96.7	8.2	8.5		
	3	5	74.34	106.2	8.2	7.7		
	4	5	76.36	109.1	5.0	4.6		
	5	5	74.83	106.9	8.5	8.0	8.1	103.1

8.4, close to the optimal value often employed for DXR (8.6), used here as the I.S. The possibility was explored of solid-phase extraction of I and II, as reported for DXR and 4'-epidoxorubicin [9–11], but the recoveries of both compounds were less than 50%. The mean recovery, using the liquid–liquid extraction conditions described above and calculated for each compound at three concentrations (0.5, 10 and 100 ng/ml) ranged from 88.5 to 95.1% and from 61.5 to 69.4% for I and II, respectively, with an imprecision of less than 11% (relative standard deviation, R.S.D.) (Table I).

A further advantage of liquid–liquid extraction is that interference from aglycone derivatives is avoided since these compounds are not back-extracted into the acidic phase. The linearity of this HPLC assay was evaluated from six separate calibration curves carried out on different days in the range 0.5–100 ng/ml by linear regression analysis of the peak-height ratio (peak height of each analyte/I.S. peak height) *versus* the concentration ratio (concentration of each analyte/I.S. concentration). The mean slope was 1.07 (R.S.D. = 7.65%) for I and 1.16 (R.S.D. = 9.56%) for II. Back-calculated standards exhibited a R.S.D. of less than 15% for both compounds. Correlation coefficients (r) ranged from 0.9975 to 1.000 for I and from 0.9958 to 0.9996 for II. When submitted to Student's t-test, intercept values were not significantly different from zero ($p > 0.05$).

The inter-day precision for I and II ($n = 25$) ranged from 7.5 to 8.4% R.S.D. and from 6.9 to 8.0% R.S.D., respectively, for concentrations between 0.7 and 70 ng/ml. At the same concentrations, the intra-day imprecision for both compounds was less than 12% R.S.D.

The accuracy, evaluated on the same plasma samples and expressed as percentage ratio of found to added amount, ranged from 100.0 to 103.1% (I, Table II) and from 98.0 to 100.5% (II).

The instrumental limit of detection for both compounds (defined as the mass of the analyte on column able to produce a signal-to-noise ratio (S/N) of 3 in absence of plasma) was 0.2 ng on column. The lower limit of detection for plasma samples was 0.5 ng/ml for both compounds (S/N > 5) (Fig. 2).

This method was applied to the determination of plasma levels of the two compounds in female rats after repeated intravenous administration of 132.9 μg/kg of I for five days. Blood samples were drawn from the abdominal aorta of the animals kept under ether anesthesia at 0.5, 1 and 4 h after

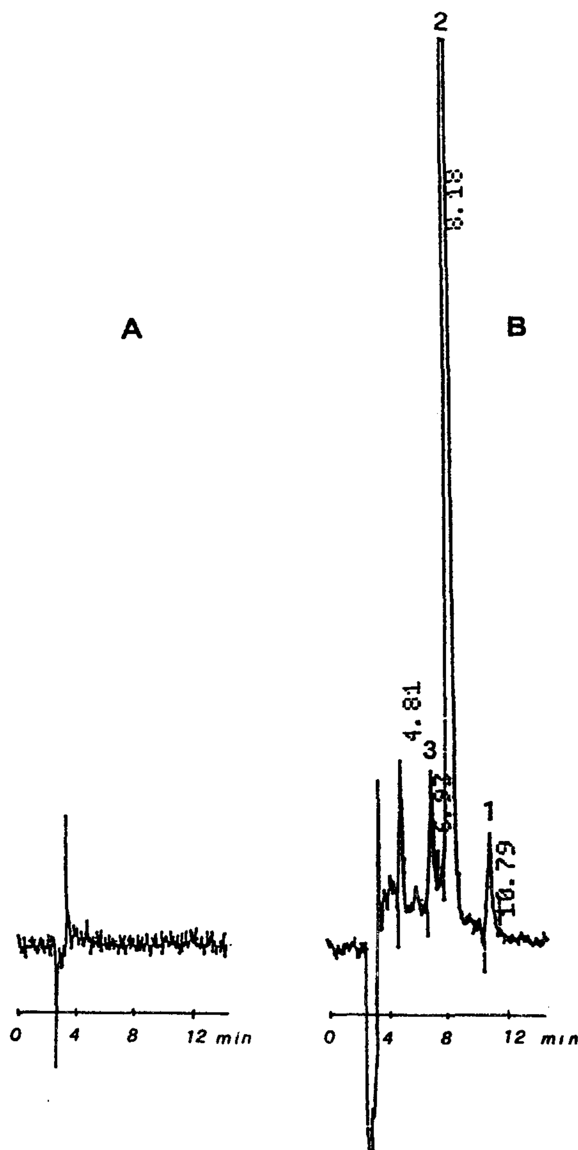

Fig. 2. Chromatograms obtained by assaying (A) 1 ml of blank human plasma and (B) 1 ml of human plasma spiked with 0.5 ng of I (peak 1), 0.5 ng of II (peak 3) and 10 ng of doxorubicin (peak 2).

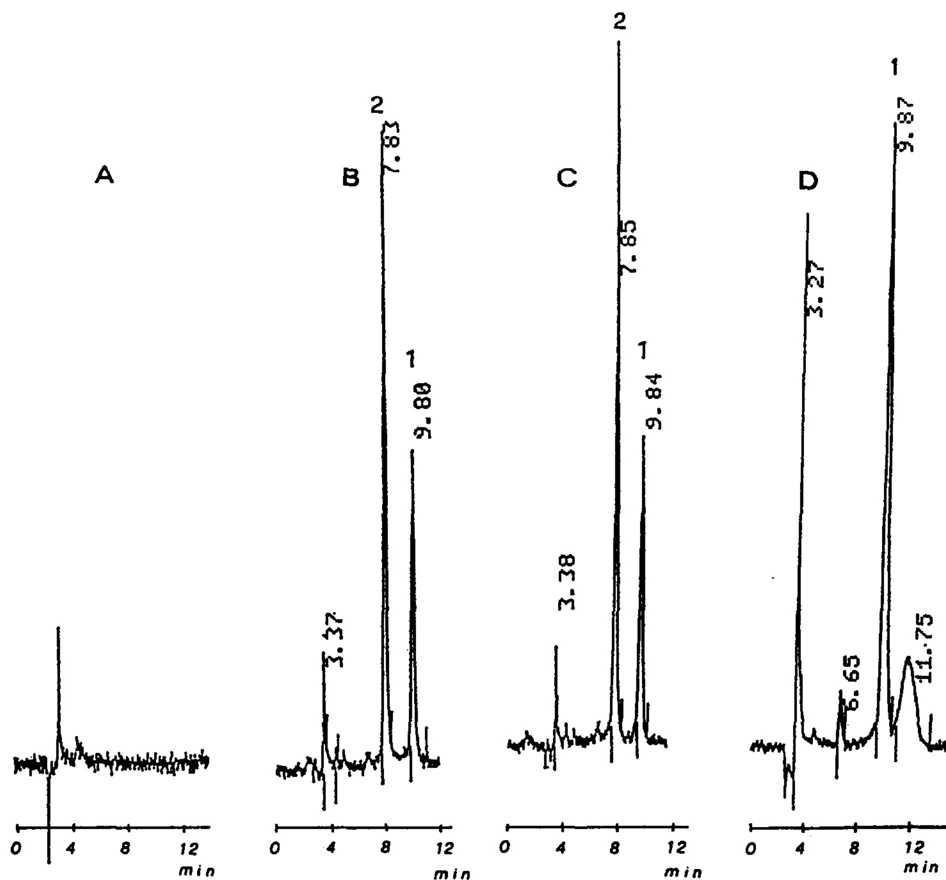

Fig. 3. Chromatograms of plasma obtained from female rats after five days of intravenous treatment with 132.9 μg/kg I per day. (A) Blank rat plasma; (B) sample collected 30 min after treatment; (C) sample collected 1 h after treatment; (D) sample collected 1 h after treatment without adding doxorubicin. Peaks: 1 = I; 2 = doxorubicin (I.S.).

administration, collected in plastic tubes at 0–4°C then immediately centrifuged at 1200 *g* for 10 min at 0–4°C. The plasma separated was stored at −20°C until assay.

Typical chromatograms obtained from these *in vivo* samples are shown in Fig. 3. Compound I is the only compound detected in rat plasma: the concentration of II was below the detection limit of the method at all test times. One sample of plasma of rats treated with I was also analysed without adding the I.S. DXR in order to check whether some DXR might have been formed by metabolism of I. No DXR was found.

CONCLUSIONS

The method described here is selective for the determination of I and its possible metabolite (II) in plasma. It proved to be linear, precise and capable of accurately quantifying the two substances in the concentration range 0.5–100 ng/ml. This method is the first analytical methodology developed that can be used for studies evaluating plasma disposition of the new compound in animals and in humans.

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